Is Standardization of Nerve Graft Groups Necessary for Experimental Studies?

Deneysel Çalışmalarda Sinir Grefti Gruplarının Standardizasyonu Gerekli midir?

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ABSTRACT Objective: Experimental studies regarding nerve regeneration use a number of methods to compose a nerve graft group for making comparisons among various peripheral nerve repair techniques. It is unclear that whether these methods used interchangeably yield similar functional, physiological and morphological results or not. The aim of this study is to demonstrate functional and morphological results of these diverse nerve grafting techniques, thereby a possible necessity of standardization. **Material and Methods:** Thirty-two adult male Sprague-Dawley rats were divided into four groups and used. A nerve defect measuring 1.5 cm was created in the sciatic nerve of the rat, and in group one, split ends of the defect were buried into neighbouring muscles (nerve defect group). In groups two and three, resected nerve segment was sutured to its own place in its original direction (orthotopic graft group) or after reversing it 180° (inverted graft group). In group four, a sural nerve graft taken from the same leg was used to bridge the defect (distant graft group). Functional, electrophysiological and histometric results obtained at 21st week were compared among the groups in order to see whether they were similar or not. **Results.** There were significant differences among the groups. Regarding functional results, orthotopic and inverted graft groups gave superior results compared to nerve defect and distant graft groups. Most importantly, it is clear that different nerve grafting techniques can not be used interchangeably. **Conclusion:** The results of the present study suggest that the use of a constant method in order to compose a nerve graft group in experimental studies may be convenient to avoid contradictory results among the researches. In other words, standardization of nerve grafting methods in experimental studies is mandatory.

Key Words: Peripheral nerves; injuries; rats; anatomy and histology

ÖZET Amaç: Sinir rejenerasyonu ile ilgili deneysel çalışmalarda farklı sinir onarımı teknikleri arasında karşılaştırma yapmak amacıyla sinir grefti grubu oluşturmak için çeşitli yöntemler kullanılmıştır. Bu yöntemlerin biribirinin yerine kullanılmasının benzer işlevsel, fizyolojik ve morfolojik sonuçlar yaratıp yaratmadığı açık değildir. Bu çalışmanın amacı, bu farklı sinir grefti yöntemlerinin morfolojik ve fizyolojik sonuçlarını ve böylece bir standardizasyonun gerekliliğini ortaya koymaktır. **Gereç ve Yöntemler:** Dört gruba ayrılan otuziki Sprague-Dawley sıçanı kullanıldı. Sıçanların siyatik sinirlerinde 1.5 cm'lik bir sinir defekti yaratıldı ve birinci gurupta, defektin ayrılmış uçları yakınındaki kasın içine gömüldü (sinir defekti grubu). İkinci ve üçüncü grupta, kesilen sinir segmenti orijinal yönünde (ortotopik greft grubu) veya 180 derece çevrildikten sonra (tersine çevrilmiş greft grubu) kendi yerine dikildi. Dördüncü grupta, defekti doldurmak için aynı bacaktan alınan bir sural sinir grefti kullanıldı (uzak greft grubu). Yirmi birinci haftada elde edilen işlevsel, elektrofizyolojik ve histometrik sonuçlar, benzer olup olmadıklarını görmek amacıyla, birbirleriyle karşılaştırıldı. **Bulgular:** Gruplar arasında önemli farklar vardı. Fonksiyonel sonuç açısından, ortotopik ve tersine çevrilmiş greft gurupları, sinir defekti ve uzak greft guruplarına göre daha iyi sonuçlar verdiler. En önemlisi de, farklı sinir greftleme yöntemlerinin birbirinin yerine kullanılamayacağının açık olduğu görüldü. **Sonuç:** Bu çalışmanın sonuçları deneysel çalışmalarda bir sinir grefti grubu oluşturmak için sabit bir yöntemin kullanılmasının araştırmacılar arasındaki çelişkili bulguları önlemek açısından uygun olabileceğini düşündürmektedir. Diğer bir deyişle, deneysel çalışmalarda kullanılan sinir greftleme metodu için bir standardizasyon getirilmesi zorunludur.

Anahtar Kelimeler: Periferik sinirler; yaralanmalar; sıçanlar; anatomi ve histoloji

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Since nerve repair is a challenging problem in reconstructive surgery, hundreds of experimental studies have been done in the medical history. Eventually, reconstructive surgeons have came to the conclusion that primary repair is the best method when available.[1] If the cut nerve is not suitable for primary repair, nerve grafting has been proposed as the gold standard.[1] Any kind of alternative nerve reconstruction method should prove its efficiency against nerve grafting in order to become an option.

Possibly due to the fact that nerve grafts are undertaken just as tubes consisting of Schwann cells, diverse methods have been used to compose a nerve graft group for making comparisons between various peripheral nerve repair techniques. Some authors sutured the cut nerve segment to its original location to constitute a nerve graft group,[2-5] while others inverted the nerve segment before being coapted.[6-9] There are also other groups who preferred to use a distant nerve graft to bridge the gap.[10,11] Therefore it is tempting to suggest that, these different methodological approaches may lead to diverse and perhaps misleading outcomes depending on size divergence, orientation discrepancy, etc. For example, results of a new technique may demonstrate better results than those of a distant nerve graft group while the same results may be concluded to be worse when compared to a nerve graft group in which the cut nerve segment is sutured to its own place in the same direction. Therefore, in an experimental study, a new technique can be regarded as a better surgical approach compared to nerve grafting while in an alternative setting it would not.

To the best of our knowledge, in the medical literature, there is no experimental studies up to date have compared the results of different nerve grafting techniques with each other. Therefore, there is no proof showing that these different techniques give equal or similar results.

This experimental study is designed to investigate the effects of different nerve grafting approaches on functional, physiological and morphological recovery, in order to shed light into the importance of a standardized methodology in the experimental peripheral nerve reconstruction studies.

The method of choice for nerve grafting in experimental studies should represent clinical situations in order to provide relevant and beneficial data. Clinically, the use of a distant nerve graft during the reconstruction of a nerve defect is the most commonly used technique, and thus, experimental studies in which a distant nerve graft has not been preferred as the nerve graft group may yield inappropriate and clinically irrelevant results. Regarding this, any method, preferably the use of a distant nerve graft, may be accepted as a standard in experimental studies, in order to provide comparable results.

MATERIAL AND METHODS

Thirty-two adult male Sprague-Dawley rats weighing 300 to 335 g each were used in this experimental study. The study was approved by the ethical committee of Uludağ University and carried out in accordance with the European Communities Council Directive. The procedures were performed under anesthesia induced by intraperitoneal injection of 50 mg/kg thiopental sodium in order to minimize any pain or discomfort, while ether inhalation anesthesia was performed during electrophysiological studies. The animals were maintained under standard laboratory conditions and allowed free access to rat chow and water.

SURGICAL PROCEDURES

The rats underwent surgery were randomly and equally divided into four groups. Surgery was performed using microsurgical techniques with the help of a binocular operative microscope (MTX-1H+SVI; Olympus Optical Co. Ltd., Tokyo, Japan) under sterile conditions. The left sciatic nerve was exposed through a muscle splitting incision and dissected from the surrounding tissues. A 1.5 cm long segment of the nerve was cut with a scalpel blade. Then, different surgical procedures were performed in four different groups: In **Group 1** (nerve defect group, n= 8) proximal and distal ends of the defect were buried into the neighbouring muscles using 10/0 nylon stitches (Figure 1A). In

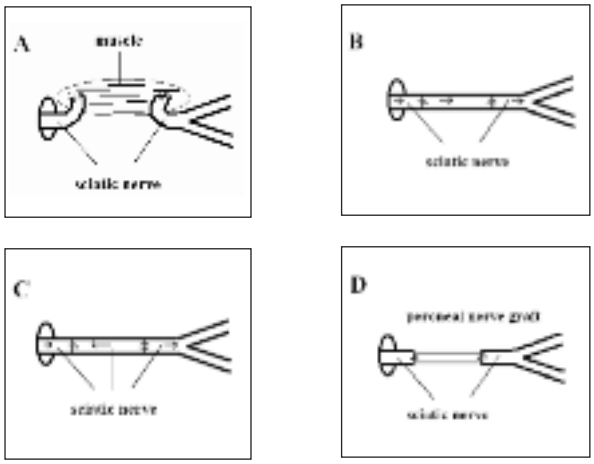

FIGURE 1: Schematic drawing of the methods used in groups. **(A)** A 1.5 cm of the sciatic nerve was resected 1 cm distal to sciatic foramen, and both ends of the sciatic nerve were buried into neighbouring muscles (nerve defect group). **(B)** Resected segment of the sciatic nerve immediately sutured to its own place in its original direction (orthotopic nerve graft group). **(C)** Resected segment was reversed, in other words, rotated 180° before being sutured (inverted nerve graft group). **(D)** Resected sciatic nerve segment was discarded and the defect was repaired by two segments of sural nerve graft taken from the same leg (distant nerve graft group).

Group 2 (orthotopic nerve graft group, n= 8) the cut nerve segment was sutured to its own place, in the same direction using four to five 10/0 nylon stitches (Figure 1B), while in **Group 3** (inverted nerve graft group, n= 8) cut nerve segment was rotated 180° before it was sutured to its own place (Figure 1C). Finally, in **Group 4** (distant nerve graft group, n= 8), the nerve defect was repaired by using a sural nerve graft that was taken from the same leg (Figure 1D). Two segments of the sural nerve graft were sutured between the ends of the nerve defect, in order to provide a better size match. Coaptations of the nerves were performed through epineural stitches. After completion of the coaptations, gluteal muscles were closed using 6/0 absorbable sutures. The skin was closed with 4/0 nylon sutures.

FUNCTIONAL TESTING

At the end of 21st week, hind paws of the animals were soaked in methylene blue solution and they were allowed to walk on a paper placed in the bottom of a walking tract to obtain foot prints as described by Özmen and collegues.[12] The procedure was repeated when an unsatisfactory result was obtained. Using the formula reported by Bain and colleagues,[13] sciatic functional index (SFI) was calculated for each animal (Table 1). An index of zero reflects normal function and an index of -100 reflects complete loss of function.

ELECTROPHYSIOLOGICAL TESTING

Following functional tests, a anesthesia induction was performed by temporary inhalation of ether. Left hind limbs of the animals were re-incised sciatic nerves were exposed and dissected carefully. In order to secure the integrity of the sciatic nerve, some muscle and scar tissues were excised with the nerve, when necessary. After exposure of the sciatic nerve, nerve conduction velocities (NCV) were measured using the MP100 data acquisition and analysis system (BIOPAC Systems Inc., Goleta, CA). During these measurements, one electrode was placed under the proximal part of the sciatic nerve (proximal to the nerve graft), and the other under the distal part of the sciatic nerve (distal to the nerve graft). Supramaximal stimulus (7 V, 0.5 m/second duration) generated by a MP100 stimulator was used to stimulate the nerve, and the distance between the electrodes was measured.

HISTOLOGICAL AND MORPHOMETRIC STUDIES

At the end of the electrophysiological testing, the rats were given a lethal dose of anesthetic and nerve tissue samples were harvested. Nerve tissue samples were taken from the sciatic nerve at the sites 0.5 cm distal to the distal suture line in all three ex-

TABLE 1: Formula used to calculate sciatic functional index.

Sciatic Functional Index (SFI) = -38.3 ([EPL – NPL] / NPL) + 109.5 ([ETS - NTS] / NTS) + 13.3 ([EIT - NIT] / NIT) – 8.8

EPL: Experimental print length (distance from the heel to the third toe), NPL: Normal print length, ETS: Experimental toe spread (distance from the first to the fifth toe), NTS: Normal toe spread, EIT: Experimental intermediary toe spread (distance from the second to the fourth toe), NIT: Normal intermediary toe spread.

periment groups (groups 2, 3 and 4), except for nerve defect group, since it was not possible to obtain any nerve tissue between the ends of the defect in that group. Additionally, corresponding samples were obtained from the normal sciatic nerve of the un-operated side and used as the control group to obtain normative data.

Tissues harvested were fixed in 4% glutaraldehyde in 0.1-M phosphate buffer at pH 7.4. Each sample was then postfixed with 1% OsO_4 in 0.1 M phosphate buffer for two hours, dehydrated through a graded series of ethanols, and embedded in Spurr's resin (Agar Scientific, Stansted, UK). Semithin sections (0.5 μm) of the entire nerve perpendicular to the long axis of the nerve fibers were then obtained, stained with a mixture of 1% toluidine blue and 1% borax in distilled water.

A Sony Cybershot DSC-F717 digital camera attached to the Nikon 4S-2 Alphaphot light microscope and Scion Image software (public domain) were used to capture and analyze images. The image analysis system was calibrated using a hemocytometer prior to all measurements and all axonal counts and measurements were performed by a single investigator (M.A.K.), who was blinded to the identity of the specimens. The first step of morphometric analysis consisted of identifying and capturing the entire fascicle image (magnification: objective 4X, optovar 1.6X, camera 0.5X), followed by measurements of the fascicle perimeter, fascicle area, minimum and maximum fascicle diameters by contouring its internal epineural edge with the mouse. The next step consisted of capturing eight sequential inner areas of the fascicle (magnification: objective 100X, optovar 1.6X, camera 0.5X), selected randomly for each nerve sample for accurate recognition, counting and analysis of the myelinated nerve fibers. A counting frame of known area (0.00175 mm^2) was then created using Scion Image software and superimposed on the digital image to be counted; myelinated axons were quantified according to the unbiased counting rule and area densities of myelinated axons (number of axons per millimeter square) were calculated. Total number of the myelinated axons was then estimated by multiplying the axonal density by total cross-sectional fascicle area estimated for each animal. As the final step, minimal axon and fiber (axon+myelin) diameter, axon and myelin sheath area of each axon with a clearly identifiable myelin sheath observed in each micrograph, were measured using Scion Image's tracing and analyzing feature (Figure 2A). Finally, G ratios and the ratio of axonal diameter to the fiber diameter were estimated for each myelinated axon.

STATISTICAL ANALYSIS

One way analysis of variance (ANOVA) was employed to compare sciatic functional indices and nerve conduction velocities. Posthoc pair-wise comparisons between individual groups were made using Tukey Test.

Normality of all morphometric data distribution was verified with Kolmogorov–Smirnov's test. Data presenting a normal distribution (fascicle perimeter, fascicle area, minimum and maximum fascicle diameters) were compared between groups by one-way analysis of variance, followed by the Tukey posttest. Data without a normal distribution (axon density, total number of axons, axon and myelin sheath area, axon and fiber diameter, G ratio) were compared between groups using one-way analysis of variance by Ranks, followed by the Mann Whitney-U posttest. Differences were considered to be significant for $p < 0.05$.

RESULTS

FUNCTIONAL EVALUATION

In nerve defect group, SFI results were close to -100, reflecting a considerable decrease in function. The mean value for SFI was -92.46 ± 2.82 in this group. Orthotopic nerve graft group demonstrated best functional results with a mean value of -56.93 ± 3.58. In inverted nerve graft group, the mean value was -67.50 ± 4.79 which represented a good functional recovery. Lastly, in distant nerve graft group, the mean value was -91.58 ± 3.31 (Table 2). The differences between the nerve defect and distant nerve graft groups as well as the orthotopic and distant nerve graft groups were not statistically significant. However, SFI values were found to be

TABLE 2: Mean sciatic functional indices and nerve conduction velocities in all groups.

Groups	Sciatic Functional Index	Nerve Conduction Velocity (m/sec)
Nerve defect group	-92.46 ± 2.82 (7.97)	Not measurable
Orthotopic graft group	-56.93 ± 3.58 (10.13)	47.68 ± 1.71 (4.19)
Inverted graft group	-67.50 ± 4.79 (13.56)	34.48 ± 1.88 (4.98)
Distant graft group	-91.58 ± 3.31 (9.37)	33.74 ± 0.87 (2.32)

Values represented as means ± the standard error of the means (SEM).

significantly improved in both orthotopic and inverted nerve groups compared to both nerve defect and distant nerve graft groups ($p< 0.001$, for all comparisons).

ELECTROPHYSIOLOGICAL EVALUATION

Since any notable nerve tissue could not be determined between the ends of the nerve defect, NCV velocity measurement was not held in nerve defect group. In orthotopic nerve graft group, the mean NCV was 47.68 ± 1.71 m/sec, while in inverted and distant nerve groups these values were 34.48 ± 1.88 m/sec and 33.74 ± 0.87 m/sec, respectively (Table 2). Best results were achieved in orthotopic graft group and NCV were found to be significantly higher compared to both inverted and distant graft groups ($p< 0.001$ for both comparison). However, there was no significant difference in NCV values of inverted and distant nerve graft groups.

HISTOLOGICAL EVALUATION

Histological appearances of all sciatic nerves obtained from the un-operated side were normal, with a similar distribution of small and large diameter myelinated nerve fibers, Schwann cells, fibroblast nuclei, blood vessels and mast cells into one, two or more fascicles, each surrounded by a well-defined perineurium (Figure 2B). Histological appearances of orthotopic (Figure 2C) and inverted (Figure 2D) nerve graft groups appeared similar and qualitative assessment of these two groups revealed preserved epineurium and perineurium, and numerous small sized myelinated axons dispersed between scarce large myelinated axons. Some myelinated fibers with a smaller diameter showed a distinctive compartmentation suggesting an active axonal regeneration and groups of extra-fascicular myelinated axons were also observed (Figure 2E) in these two groups, while blood vessels appeared normal. In the distant nerve graft group (Figure 2F) a moderate quantity of fibers with smaller diameter and less distinctive myelin sheath were observed. Myelinated axons in this group usually formed intra and extra-fascicular compartmentation and demonstrated few focal areas of continued Wallerian degeneration. Axonal atrophy and increased cellular activity were also observed in the distant nerve graft group, while almost no discernable myelinated axons with large diameters were encountered within the fascicles.

MORPHOMETRIC EVALUATION

Axonal Density and Total Axon Numbers

Histomorphometrical evaluations revealed that the axonal density was significantly higher in the orthotopic and inverted nerve graft groups compared to normal sciatic nerve group ($p< 0.001$ for both comparisons) and the distant nerve graft group ($p< 0.001$ for both comparisons), while no significant difference was found between the orthotopic and inverted nerve graft groups (Figure 3). Axonal density in the distal part of the distant nerve graft group was also significantly higher compared to normal sciatic nerve group.

When total numbers of the myelinated axons were estimated by multiplying the whole cross-sectional area of the individual nerves with the axonal densities, orthotopic and inverted nerve graft groups had a significantly higher number of myelinated axons (Figure 4) compared to both normal sciatic nerve group and distant nerve graft group ($p< 0.001$ for all comparisons), while there were no significant differences in total number of myelinated axons between the orthotopic and inverted nerve graft groups. The total number of myelinated nerve fibers was significantly ($p< 0.001$) smaller in distant nerve graft group compared to the normal sciatic nerve group.

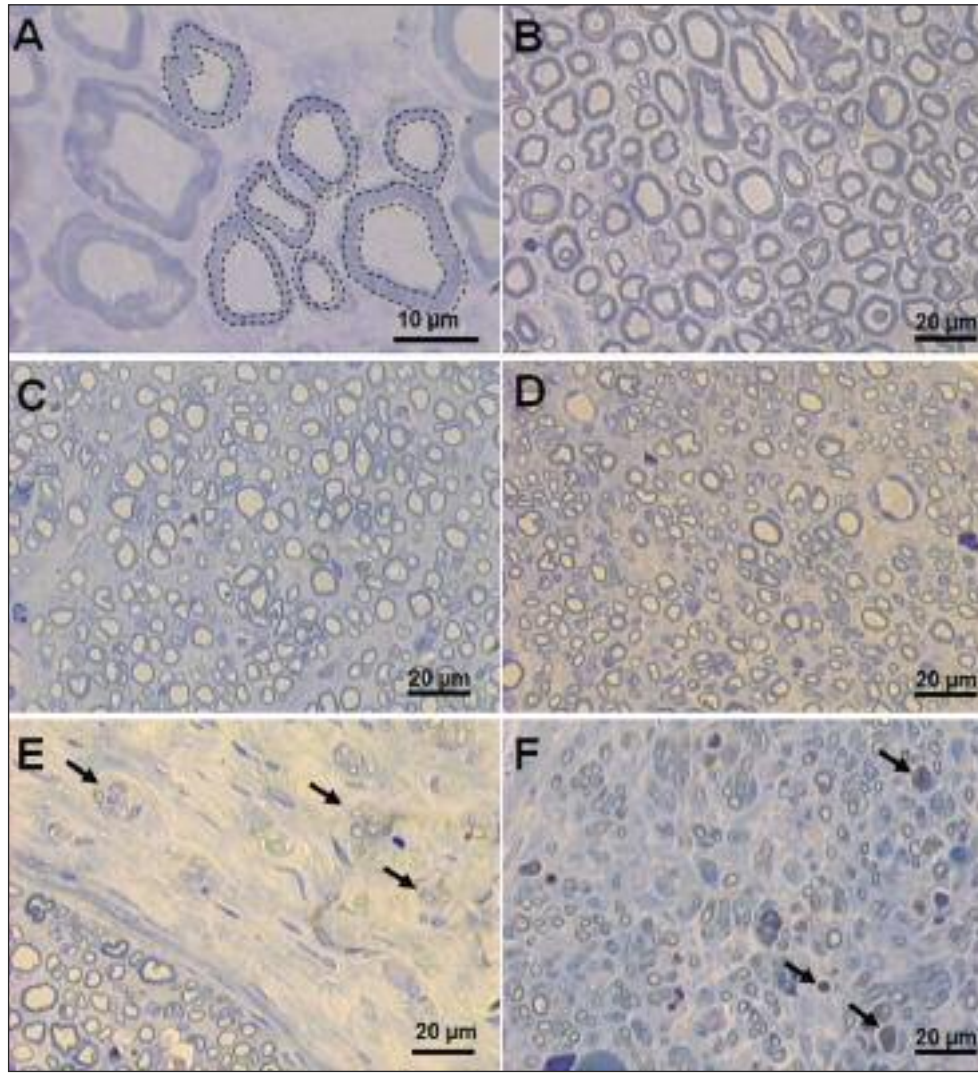

FIGURE 2: (A) Shows examples of myelinated axons which were traced and analyzed for axon and fiber diameters and axon and myelin sheath areas using computer automated morphometry software. Photomicrographs of the sciatic nerve of normal nerve group **(B),** orthotopic **(C)**, inverted **(D)** and distant **(F)** nerve graft groups. Small and large sized myelinated fibers are distributed regularly in the normal nerve group **(B)**, while numerous smaller myelinated axons were dispersed among scance large axons in orthotopic **(C)** and inverted **(D)** nerve graft groups. Moderate quantity of fibers with even more smaller diameters and less prominent myelin sheath are observed, together with few focal areas of continuing Wallerian degeneration (arrows) in distant nerve graft group **(F)**. In Figure **2E**, examples of extra-fascicular myelinated axons (arrows) are shown.

Fascicle Area, Perimeter, Minimum and Maximum Diameters

Results of the comparisons for entire fascicle area, perimeter, minimum and maximum diameters of the segments distal to nerve grafts in three nerve graft groups and the un-operated sciatic nerve group are shown in Table 3. Entire fascicle areas of nerve segments distal to grafts were significantly smaller in orthotopic ($p< 0.01$), inverted ($p< 0.001$) and distant ($p< 0.001$) nerve graft groups compared to normal sciatic nerve group. Mean fascicle area of distant nerve graft group was also significantly smaller compared to orthotopic nerve graft group ($p<0.05$), while there was no significant difference between the orthotopic and inverted, and between the inverted and distant nerve graft groups. Regarding all of the four parameters above, there were no differences between the orthotopic and inverted nerve groups. Fascicle perimeters of the orthotopic ($p< 0.05$), inverted ($p< 0.01$) and distant ($p< 0.001$) nerve groups were significantly smaller than the normal nerve group. Minimal fascicle diameters of the inverted ($p< 0.01$) and distant ($p< 0.01$) nerve

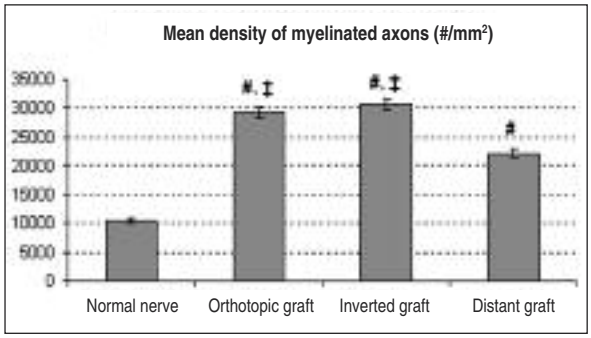

FIGURE 3: A bar diagram showing the mean density (±SEM) of myelinated axons in normal nerve, orthotopic, inverted and distant nerve graft groups (n = 8, for all groups). # and ‡, represent differences compared to normal nerve (#, p<0.001) and distant nerve graft (‡, p<0.001) groups, respectively.

graft groups were also significantly smaller than the normal nerve group. Regarding maximum fascicle area, inverted ($p< 0.05$) and distant ($p< 0.001$) nerve graft groups yielded significantly smaller areas compared to the normal nerve graft group. The differences between the orthotopic and distant nerve graft groups, and inverted and distant nerve graft groups were also significant ($p< 0.001$ and $p< 0.05$, respectively). Mean values and statistical comparisons for these data are summarized in Table 3.

Fiber and Axon Diameters, G Ratios

The mean minimum diameter of myelinated fibers was 7.08 μm (range: 1.22-20.15 μm) in the normal sciatic nerve group while for orthotopic, inverted and distant nerve graft groups it was found as 3.62 μm (range: 1.41-10.56 μm), 3.09 μm (range: 1.05-10.6 μm) and 2.88 μm (range: 1.07-7.68 μm), respectively. The average minimal diameter of myelinated fibers was significantly higher in the normal sciatic nerve group compared to orthotopic, inverted and distant nerve graft groups ($p< 0.001$, for all comparisons). Significant differences were observed among the experiment groups. The average minimum diameter of myelinated fibers was significantly higher in the orthotopic nerve graft group compared to both inverted and distant nerve graft groups ($p< 0.001$ for both comparisons), while it was also found to be significantly greater in the inverted nerve graft group compared to distant nerve graft group ($p< 0.01$). The histograms of the frequency distribution of fibers according to diameter (Figure 5) showed that in normal sciatic nerve group, fibers presented a normal distribution, with their diameters ranging between 1.5 and 20 μm and a peak between 5 and 7 μm, which is the usual pattern for the sciatic nerve of rats. In orthotopic nerve graft group, there was a trend for a left deviation and unimodal distribution, with fiber diameter ranging from 1 to 11 μm and peaking at 2-4 μm. A similar configuration was repeated in inverted nerve graft group, in which the peak was at 2-4 μm for a similar diameter range. In distant nerve graft group, range and peak were similar, but distribution was clearly unimodal and frequency was much higher (43.4%) for small diameter fibers (between 2 and 3 μm).

The mean minimum axon diameter was 4.39 μm (range: 0.57-14.13 μm) in the normal sciatic nerve group while in orthotopic, inverted and distant nerve graft groups it was 2.25 μm (range: 0.36-9.95 μm), significantly higher in the normal sciatic nerve group compared to orthotopic, inverted and distant nerve graft groups ($p< 0.001$, for all

TABLE 3: Comparison of area, perimeter, minimum and maximum diameters of entire fascicle of sciatic nerve in normal sciatic nerve, orthotopic, inverted and distant nerve graft groups

Groups	Fascicle area (mm^2)	Fascicle perimeter (mm)	Maximum fascicle diameter (mm)	Minimal Fascicle Diameter (mm)
Normal nerve	1.0675 ± 0.044	3.7275 ± 0.071	1.2713 ± 0.196	1.0113 ± 0.2943
Orthotopic graft	0.6688 ± 0.564 $^{\#\#,\ddagger}$	3.3825 ± 0.159‡	1.2350 ± 0.055‡‡	0.8063 ± 0.089
Inverted graft	0.5913 ± 0.695$^{\#\#\#}$	2.9300 ± 0.154$^{\#\#}$	1.0900 ± 0.052$^{\#,\ddagger}$	0.6725 ± 0.062$^{\#\#}$
Distant graft	0.4038 ± 0.091$^{\#\#\#}$	2.6363 ± 0.148$^{\#\#\#}$	0.8769 ± 0.445$^{\#\#\#}$	0.6250 ± 0.72$^{\#\#}$

Values represented as means ± the standard error of the means [SEM] $^{\#}$; $p< 0.05$, $^{\#\#}$; $p< 0.01$ and $^{\#\#\#}$; $p< 0.001$ represent significance compared to normal nerve group, while ‡; $p<0.05$, ‡‡; $p<0.01$ and ‡‡‡; $p< 0.001$ represents significance compared to distant graft group. There were no significant differences between the orthotopic and inverted nerve groups for any of the four parameters.

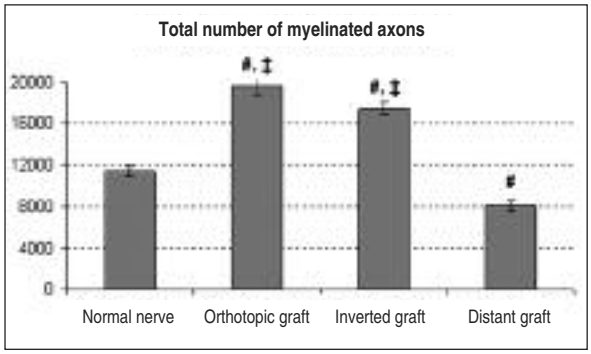

FIGURE 4: A bar diagram showing the total number (±SEM) of myelinated axons in normal nerve, orthotopic, inverted and distant nerve graft groups (n = 8, for all groups). # and ‡, represent differences compared to normal nerve (#, p<0.001) and distant nerve graft (‡, p<0.001) groups, respectively.

comparisons). The mean minimum diameter of myelinated fibers was significantly greater in the orthotopic nerve graft group compared to both inverted (p< 0.05) and distant (p< 0.001) nerve graft groups, while no significant difference was present between the inverted and distant nerve graft groups. The histograms of the frequency distribution of axons with respect to diameter (Figure 6) showed a normal distribution for the normal sciatic nerve group, with diameters ranging between 0.5 and 15 μm and peaking at 3-4 μm. In orthotopic nerve graft group, the distribution presented a left deviation, with the axon diameters ranging between 0.5 and 9.5 μm and peaking at 1-2 μm. The same configuration was repeated in inverted and distant nerve graft groups, both with a peak at 1-2 μm, thus indicating a clear predominance of small diameter axons.

The average *G* ratio was 0.68 (range: 0.36-1) for the nerves of the normal sciatic nerve group, while in orthotopic, inverted and distant nerve graft groups it was 0.68 (range: 0.31-0.97), 0.80 (range: 0.36-1.15) and 0.74 (range: 0.42–1.34) respectively, with a significant difference between the normal sciatic nerve group and orthotopic (p< 0.05), inverted (p< 0.001) and distant (p< 0.001) nerve graft groups. The histograms of the frequencies of the *G* ratio (Figure 7) showed a normal distribution for the normal sciatic nerve group, ranging from 0.3 to 1 with a peak at 0.6-0.7. In orthotopic nerve graft group, distribution tended to be similar to the normal sciatic nerve, but with a very small variation between ratio frequencies, with values ranging from 0.1 and 0.9 and a peak between 0.6 and 0.8. In inverted and distant nerve graft groups, *G* ratio distribution presented a strong right deviation, with a peak at 0.7-0.8, and contained higher number of axons with a G ratio of 0.8-0.9.

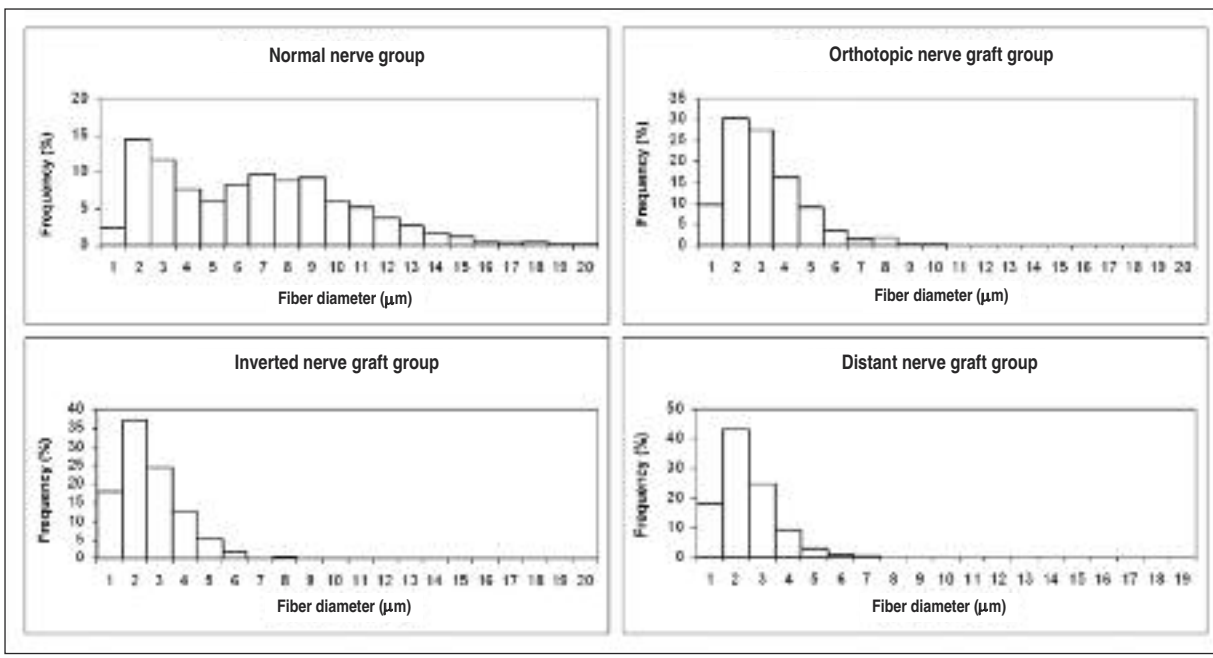

FIGURE 5: The histograms of the frequency distribution of fibers according to diameter.

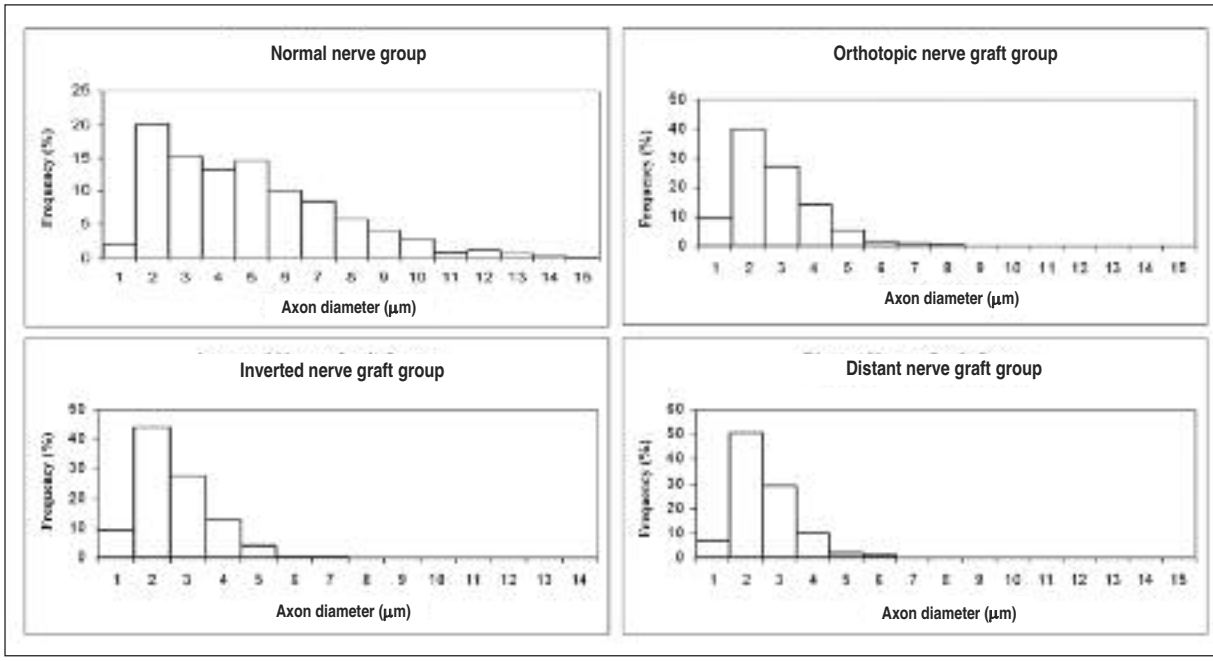

FIGURE 6: The histograms of the frequency distribution of axons according to diameter.

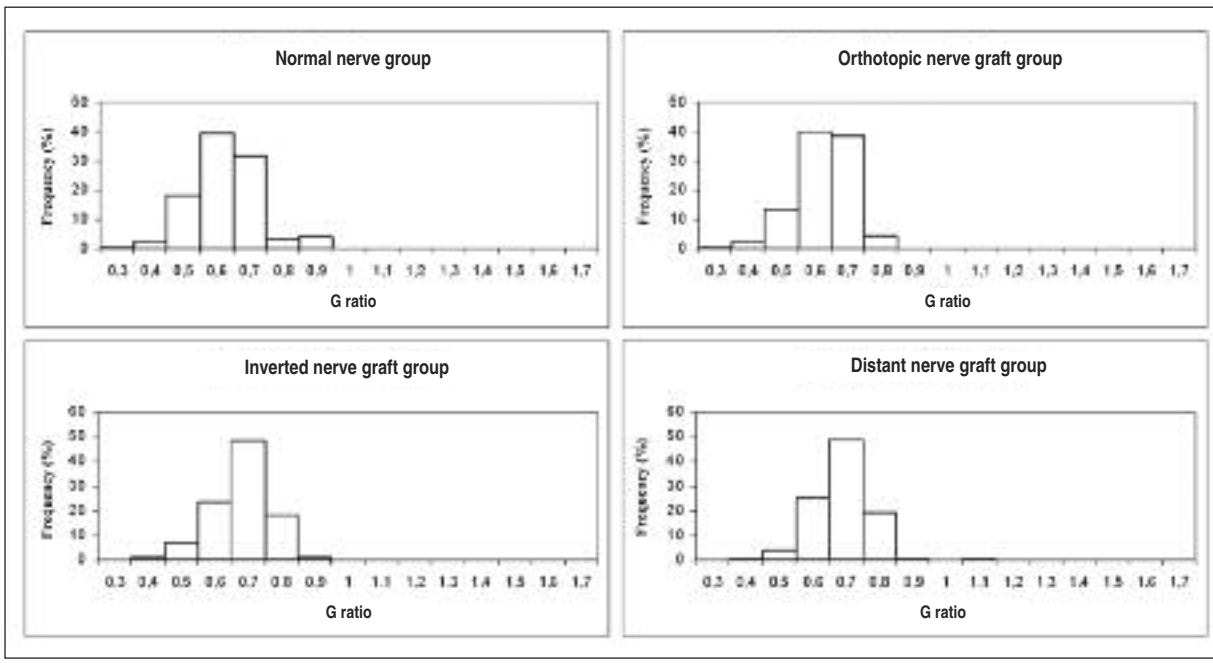

FIGURE 7: The histograms of the frequency distribution of G ratio.

Axon and Myelin Sheath Area

The average axon area was 32.92 μm^2 (range: 0.62–340.41 μm^2) for the nerves of the normal sciatic nerve group, while for orthotopic, inverted and distant nerve graft groups it was 8.77 μm^2 (range: 0.61-65.26 μm^2), 7.37 μm^2 (range: 0.45-61.90 μm^2) and 6.12 μm^2 (range: 0.44-38.39 μm^2), respectively. The average axon area was significantly higher in the normal sciatic nerve group compared to orthotopic, inverted and distant nerve graft groups (p<

0.001 for all comparisons). The mean axon area was also significantly greater in the orthotopic nerve graft group compared to both inverted ($p< 0.01$) and distant ($p< 0.001$) nerve graft groups, and it was also found to be significantly greater in the inverted nerve graft group compared to distant nerve graft group ($p< 0.001$).

The mean myelin sheath area was 39.88 μm^2 (range: 0.65–329.41 μm^2) in the normal sciatic nerve group, while for orthotopic, inverted and distant nerve graft groups it was 10.29 μm^2 (range: 1.44-92.46 μm^2), 6.17 μm^2 (range: 0.26-32.20 μm^2) and 5.00 μm^2 (range: 0.20-60.90 μm^2), respectively. The mean myelin sheath area was significantly greater in the normal sciatic nerve group compared to orthotopic, inverted and distant nerve graft groups ($p< 0.001$, for all comparisons). The mean myelin sheath area was significantly greater in the orthotopic nerve graft group compared to both inverted and distant nerve graft groups ($p< 0.001$, for both comparisons). It was also found to be significantly greater in the inverted nerve graft group compared to distant nerve graft group ($p< 0.001$).

DISCUSSION

Knowledge about nerve regeneration is the key point for success in peripheral nerve surgery. Therefore, considerable amount of experimental and clinical studies that investigate peripheral nerve regeneration have been designed and performed. Since clinical studies have their limitations, experimental studies are very important in order to accumulate data on the subject. In review of the medical literature, it is seen that rat model is the most commonly used one to study peripheral nerve regeneration. In general, the results of every newly developed peripheral nerve repair technique has been usually compared to those obtained from a variety of alternative techniques, one of which almost always being the nerve graft group. Unfortunately, nerve graft groups used in these studies are not homogenous and three main alternative methods have been preferred to compose a nerve graft group; (i) cutting a segment of nerve and immediately suturing it to its own place in its original direction, (ii) reversing the nerve segment before suturing, and (iii) using a distant nerve graft to reconstruct the nerve defect. All of these three methods have been regarded as nerve graft group, and the author decided one of them. This discrepancy between the studies raises important questions such as "Does choosing different methods affect the results of the study and influence or even bias the success of the tested method?" This experimental study was designed to provide insight and seek answers for this question.

Three common methods of nerve grafting were evaluated by means of electrophysiology, functional assessment and histomorphometry. Among them, functional assessment performed using walking tract analysis is accepted to be the most important one,[13-15] since functional index is considered as an assessment of overall nerve function since walking requires complex motor-unit reinnervation coordinated by cortically integrated sensory feedback.[14,15] In orthotopic and inverted graft groups, functional results were significantly better than those of nerve defect and distant nerve graft groups. The reason for this result may be explained by good size match between the coapted nerve ends in orthotopic and inverted graft groups, although in distant nerve graft group two segments of nerve graft were used to provide better size match between the graft and the sciatic nerve. It is well known that, size match between the graft and the cut nerve, as well as the consistency in topographic orientation of the fibers, is an important factor in nerve regeneration.[1] Two segments of nerve graft may provide size match, but physically may not cover all the fibers those will sprout during the regeneration process. Eventually, some of the fibers will be lost or misdirected. It is also difficult to provide consistency in topographic orientation between a distant nerve graft and the reconstructed nerve.

Our results revealed significantly better NCV results in the orthotopic nerve graft group compared to inverted and distant nerve graft groups; although no significant difference was observed between the latter two graft groups. This result may reflect the effect of better topographic orientation between orthotopic nerve graft and the cut

nerve, which is difficult to achieve in an inverted or distant nerve graft. NCV measures the fastest conducting nerve fibers, and it is dependent on axon diameters, myelination and internodal distance.[14] However, it should be noted that NCV does not evaluate total nerve function, because a nerve may have only a few fibers that conduct very well even though the majority of remaining fibers are damaged.[14]

In addition to functional assessment and NCV measurements, a detailed histologic and histomorphometric study was performed, in order to evaluate the effects of diverse grafting methods on morphology of the repaired peripheral nerve, and to shed light into the cellular basis of functional recovery. In the present study, endpoint (21 week) histomorphometry demonstrated that the total number and density of myelinated fibers that regenerated were significantly higher in the orthotopic and inverted graft groups compared to distant nerve graft group, and this may explain better functional recovery observed in these two groups compared to distant nerve graft group.

Apart from three alternative graft groups, normal sciatic nerves from the un-operated side of the animals were harvested in order to be used as controls in the morphometric studies. When compared with these control sciatic nerves, axonal density in the distal part of the sciatic nerve was found significantly higher in all three graft groups . However, the distal myelinated axon and fiber diameters as well as axon and myelin sheath areas were significantly smaller in all graft groups compared to these normal sciatic nerves. Owing to its critical functional role in the peripheral nervous system, measurement of axon and fiber diameter distribution is an important, and the mean fiber diameter, myelin thickness and myelin area not only provide measures of the maturity for the regenerating nerve fibers but also directly effect nerve function, and they are directly correlated with NCV.[16-18] Therefore, apart from estimating axonal density and total axon numbers, other morphometric parameters including minimum axon and fiber (axon+myelin) diameter, axon and myelin sheath area and G ratios (the ratio of axonal diameter to the fiber diameter) were estimated in order to provide information on the maturation of regenerating axons and their relevance with the functional recovery. The morphometric results of this study revealed significant results that may correspond to the SFI and NCV recorded in three experimental nerve graft groups. For example, the mean diameter of myelinated fibers and myelin sheath area were found to be significantly greater in the orthotopic nerve graft group compared to both inverted and distant nerve graft groups. Similarly, significantly greater values were observed in the inverted nerve graft group compared to distant nerve graft group. Therefore, it is tempting to suggest that these morphological findings correspond to the SFI and NCV results obtained from these three groups, since both morphological parameters have been regarded as measures of axonal maturity and determinants of nerve function and NCV.

Following immediate neurorrhaphy or nerve grafting, axons reenter the distal stump and regenerate down the perineurial and endoneurial tubes,[19] but the incomplete return in fiber diameter seen may compromise subsequent conductive[20] and functional[21] properties. We documented here that in distant nerve graft group, the distribution of fiber diameter was clearly unimodal and frequency was much higher (43.4%) for small diameter fibers (between 2 and 3 μm) compared to those observed in orthotopic and inverted nerve graft groups. Therefore, apart from decreased number of myelinated axons, reduced fiber diameter may also be responsible for the poorer functional results obtained in the distant nerve graft group.

Consequently, consistency in methodology is important in experimental animal studies. Therefore, homogeneity is inevitable. In order to get homogeneity, similar or same methods should be used to compose a definite group. Results of this experimental study show that, there are noticeable differences among the methods used to compose a nerve graft group during experimental studies, although they are used interchangeably. The authors believe that, this heterogeneity affect the results of the study. Therefore, a standard method should be de-

scribed to compose a nerve graft group in experimental studies. This method should preferably be distant nerve graft method, since it may better reflect clinical situations.

CONCLUSION

Different methods have been used to compose a nerve graft group in experimental studies in the medical literature. In this experimental study, frequently used methods have been compared and the results show that different methods yield different results. Therefore, a standardization is necessary.

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